

Effects of polyunsaturated fatty acids on calcium response and degranulation from RBL-2H3 cells

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Abstract

To investigate the biological activity of various polyunsaturated fatty acids (PUFAs) on the allergic reaction, we examined the effects of six PUFAs and two saturated fatty acids on calcium response and degranulation from rat basophilic leukemia (RBL-2H3) cells. Between 20 and 40 μ M of six PUFAs (ω -6 series: arachidonic acid [AA, C20:4], γ -linolenic acid [γ -LN, C18:3] and linoleic acid [LA, C18:2]; ω -3 series: α -linolenic acids [α -LN, C18:3] and eicosapentaenoic acid [EPA, C20:5]; and ω -9 series: oleic acid [OLE, C18:1]), or two saturated fatty acids (stearic acid [STA, C18:0] and arachidic acid [AD, C20:0]) were used to examine the effects on calcium response and degranulation from RBL-2H3 cells. Calcium response was monitored using the fluorescent calcium indicator fura-2, while degranulation was monitored by measuring histamine release from the cells. Three ω -6 PUFAs (AA, α -LN and LA) dose-dependently increased the cytosolic free-calcium concentration and histamine release from RBL-2H3 cells. This phenomenon was specific to the ω -6 PUFAs, the ω -3 PUFAs (α -LA and EPA), ω -9 PUFA (OLE) and the saturated fatty acids (STA and AD) had no effect. The increase in the cytosolic free-calcium concentration caused by the ω -6 PUFAs depended on the existence of external calcium, cell viability and the cellular IP₃ levels remained unchanged throughout the experiment. These results suggest that ω -6 PUFAs work as direct mediators of calcium signaling pathways in RBL-2H3 cells.

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1. Introduction

Linoleic acid and α -linolenic acid are essential polyunsaturated fatty acids, the former a ω -6 and the latter a ω -3 fatty acid [1,2]. In mammalian cells, linoleic acid is converted to arachidonic acid by δ -6 desaturase; α -linolenic acid is also converted to eicosapentaenoic

acid (EPA) by δ -6 desaturase and is then further converted to docosahexaenoic acid (DHA). Recently, ω -3 fatty acids have attracted a great deal of attention because of their anti-atherosclerotic, anti-inflammatory and anti-allergic properties [1,3,4]. Many in vivo experiments have suggested that diets containing large amounts of arachidonic acid and the fatty acid precursors of the ω -6 family might promote atherosclerosis and inflammation, while diets containing a high amount of ω -3 PUFAs might protect against these conditions [3,4].

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Some studies concerning the mechanisms of PUFA action have been carried out using cultured cells; however, few in vitro studies on the direct effects of PUFAs on mast cells or basophils have been performed, and the molecular mechanisms by which PUFAs regulate mast cell functions are largely unknown.

Rat basophilic leukemia (RBL-2H3) cells have been used as in vitro systems for the analysis of cellular and molecular mechanisms involved in the secretion of histamine and serotonin by exocytosis [5,6]. RBL-2H3 cells have the same functions as primary mast cells and normal basophils which mediate allergic type I reaction [5,6]. Therefore, in the present study, we studied the effects of several PUFAs on RBL-2H3 cell signal transduction to determine the biological effects of these PUFAs on allergic reactions.

2. Materials and methods

2.1. Reagents

The PUFAs (arachidonic acid [AA], γ -linolenic acid [γ -LN], linoleic acid [LA], α -linolenic acids [α -LN], eicosapentaenoic acid [EPA] and oleic acid [OLE]) and saturated fatty acids (stearic acid [STA] and arachidic acid [AD]) were obtained from the following companies; AA: Funakoshi (Tokyo, Japan), γ -LN and LA: Sigma (St. Louise, MO, USA), OL, SA and AD: Wako Pure Chemical Industries (Osaka, Japan), and α -LN and EPA: Idemitsu Petrochemicals (Tokyo, Japan). A 20-mM ethanol stock solution of each PUFA was made, and stored at -70°C .

Fura-2-AM was obtained from Dojindo (Kumamoto, Japan). An IP_3 assay kit was obtained from Amersham Radiochemical (Amersham, UK).

2.2. Cells

All experiments were performed using a secreting subline of rat basophilic leukemia cells, RBL-2H3 [5]. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM),

supplemented with 10% fetal calf serum, as described previously [6].

2.3. Pipes buffer

The Pipes buffer that was used had the following composition: 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl_2 , 1.0 mM CaCl_2 , 5.5 mM glucose and 10 mM piperazine-*N,N'*-bis (2-ethanesulfonic acid) (Pipes; pH 7.4). For the experiments performed in the absence of external calcium, Pipes-EGTA buffer (14 mM NaCl, 5 mM KCl, 1.0 mM EGTA, 5.5 mM glucose and 10 mM Pipes; pH 7.4) was used.

2.4. Measurement of cytosolic free-calcium concentration ($[\text{Ca}^{2+}]_i$)

RBL-2H3 cells (6×10^5 cells/ml) were loaded with fura-2-AM (6 μM), as described previously. After centrifugation to remove free dye, the cells were resuspended in 1.5 ml of buffer. Fura-2-loading under the above conditions was adequate to monitor the changes in $[\text{Ca}^{2+}]_i$. The resulting fluorescence was measured in a 1 cm quartz cuvette using a Shimadzu RF-5000 spectrophotometer (excitation, 335 or 362 nm; emission, 500 nm) with stirring at 37°C . Estimation of $[\text{Ca}^{2+}]_i$ from fura-2 fluorescence data was based on the method described in a previous paper [7].

2.5. Measurement of histamine release from RBL-2H3 cells

The degranulation process was monitored by measuring histamine release, as described previously. Cells were pre-incubated for 16 h at 37°C on a 24-well microplate (Falcon, No. 3047) in 1 ml/well of DMEM containing 10% fetal calf serum. The cells were washed three times with Pipes buffer and then challenged with 500 μl of each concentration of PUFA for 35 min. The amount of histamine release was determined by HPLC according to the post-column derivatization method using ortho-phthalaldehyde as described previously [6]. The percentage of histamine release (% release) was calculated using the following formula: $100A/(A+B)$, where A is the amount of histamine released from the cells after stimulation,

Table 1
Effects of various polyunsaturated fatty acids on histamine release from RBL-2H3 cells

Drug		Histamine release (%)	Drug		Histamine release (%)	Drug		Histamine release (%)
AA	20 μM	12.31 $\pm$ 1.12*	α -LN	20 μM	8.85 $\pm$ 0.24	STA	20 μM	10.01 $\pm$ 0.47
	40 μM	36.56 $\pm$ 1.16**		40 μM	9.37 $\pm$ 0.13		40 μM	9.38 $\pm$ 1.09
LA	20 μM	11.91 $\pm$ 0.39*	EPA	20 μM	9.42 $\pm$ 0.23	AD	20 μM	9.36 $\pm$ 0.06
	40 μM	23.28 $\pm$ 0.22**		40 μM	8.51 $\pm$ 0.25		40 μM	10.34 $\pm$ 0.18
γ -LN	20 μM	12.36 $\pm$ 0.41*	OLE	20 μM	10.37 $\pm$ 0.58			
	40 μM	28.35 $\pm$ 0.38**		40 μM	11.29 $\pm$ 0.11*			

Values are the mean $\pm$ S.D. of 3 experiments.

The histamine release (HR) % from RBL-2H3 cells treated with a control solvent (0.4% EtOH) was 9.46 $\pm$ 0.50%.

Levels of significance from control were determined by the Student's *t*-test. * p <0.05, ** p <0.01.

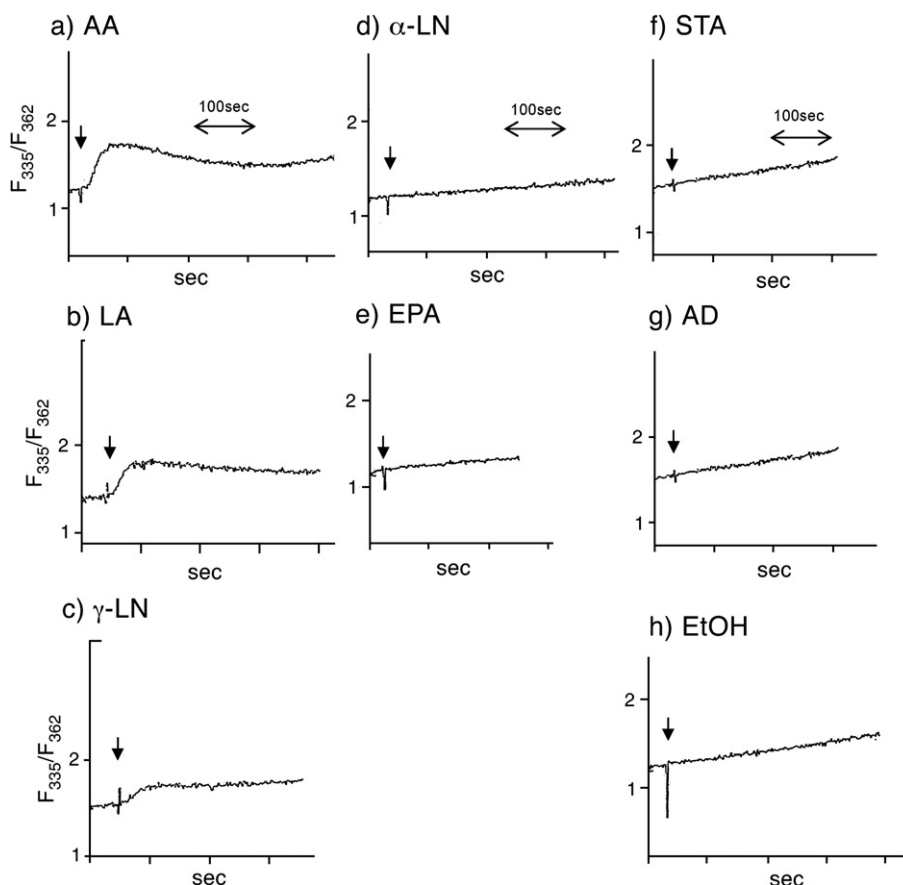


Fig. 1. Effects of PUFAs on $[Ca^{2+}]_i$ in RBL-2H3 cells. Changes in $[Ca^{2+}]_i$ in RBL-2H3 cells were monitored using the fluorescence Ca^{2+} indicator dye, fura-2. Where indicated, 40 μM of fatty acids (a, AA; b, LA; c, γ -LN; d, α -LN; e, EPA; f, STA; g, AD) or a solvent control (0.4% EtOH) was added to the cuvette, and $[Ca^{2+}]_i$ was monitored by measuring the ratio of the fluorescence intensity (F_{335}/F_{362}) of fura-2. The traces are representatives of typical experiments that were repeated at least three times.

and B is the amount of residual histamine remaining in the cells after stimulation.

2.6. Measurement of IP_3 formation

IP_3 formation in the RBL-2H3 cells after PUFA stimulation was measured as described previously [6,7]. Briefly, RBL-2H3 cells (8×10^6 cells/ml) were stimulated with PUFA for 20 min. The IP_3 was then extracted, and the concentration in the cells (4×10^5 cells equivalent) was measured using an IP_3 assay kit (Amersham, UK), as described previously [6].

3. Results

3.1. Effects of different PUFAs on histamine release from RBL-2H3 cells

As shown in Table 1, 20–40 μM of AA (ω -6, C20:4) induced histamine release from RBL-2H3 cells in a dose-dependent manner. We next examined the specificities of

PUFAs with different ω -numbers. As shown in Table 1, 20–40 μM of LA (ω -6, C18:2) or γ -LN (ω -6, C18:3) also induced histamine release in a dose-dependent manner. The extents of histamine release induced by 40 μM of LA or γ -LN (23.28% and 28.35%, respectively) were slightly lower than that induced by AA (36.56%). Unlike the ω -6 PUFAs, none of the ω -3 PUFAs (α -LN, C18:3 or EPA, C20:5) induced histamine release from the RBL-2H3 cells. None of the PUFA concentrations used in the study were toxic to the RBL-2H3 cells, as determined using a Wako LDH-cytotoxic test kit (data not shown). In addition, a ω -9 fatty acid, oleic acid (ω -9, C18:1), produced little or no induction of histamine release, nor did the saturated fatty acids, stearic acid (C18:0) and arachidic acid (C20:0).

From the above results, we suggest the several trends as follows. (i) The position of the double bonds relative to the terminal methyl group is important, since the ω -6 series of fatty acids was more active than the ω -3 series. (ii) The effect on histamine release requires the presence of several double bonds in a *cis* configuration and increases with the degree of

unsaturation, since neither of the saturated fatty acids produced an effect.

3.2. Effects of different PUFAs on calcium response in RBL-2H3 cells

To further examine the effects of PUFAs on histamine release from RBL-2H3 cells, we examined the effects of PUFAs on calcium response. As shown in Fig. 1a, 40 μ M of AA induced a rapid increase in $[Ca^{2+}]_i$ shortly after the addition of AA, as shown by the increase in fura-2 fluorescence; this increase in $[Ca^{2+}]_i$ was maintained for more than 300 s. As shown in Fig. 1b and c, the other ω -6 PUFAs (LA and γ -LN) also increased $[Ca^{2+}]_i$. On the other hand, as shown in Fig. 1d and e, the ω -3 PUFAs (α -LN and EPA) did not induce an increase in $[Ca^{2+}]_i$ in the RBL-2H3 cells. Furthermore, as shown in Fig. 1f and g, the saturated fatty acids, stearic acid and arachidic acid, also did not induce an increase in $[Ca^{2+}]_i$. To determine the dose-dependent and external calcium-dependent effects of AA, the increase in $[Ca^{2+}]_i$ induced by 10–40 μ M of AA was examined as shown in Fig. 2 AA increased the $[Ca^{2+}]_i$ in a dose-dependent manner, starting at a threshold of 10 μ M; the $[Ca^{2+}]_i$ induced by 20 μ M of AA was about 70% of the $[Ca^{2+}]_i$ induced by 40 μ M. The $[Ca^{2+}]_i$ increase was significantly smaller when the experiment was performed using a free- Ca^{2+} -depleted buffer. Therefore, the increase in $[Ca^{2+}]_i$ induced by AA seems to be caused mainly by calcium influx from the external medium and partly by the mobilization of Ca^{2+} from intracellular stores.

Furthermore, since the increase in $[Ca^{2+}]_i$ induced by 40 μ M of AA was slightly or not inhibited by preincubation with a cyclooxygenase inhibitor (10 μ M of indomethacin) or a lipoxygenase inhibitor (10 μ M of nordihydroguaiaretic acid [NDGA]), a direct effect of AA on ion channels of RBL-2H3 cells was suggested (data not shown).

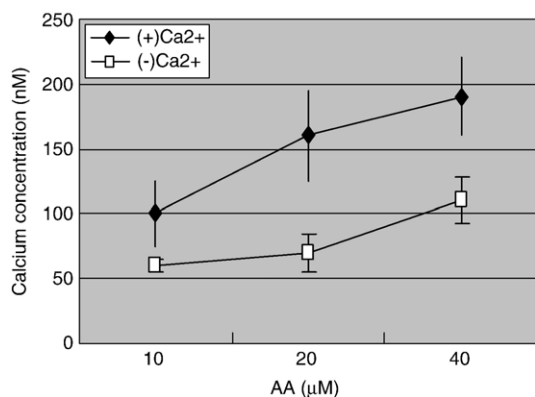


Fig. 2. Effects of various concentrations of AA on $[Ca^{2+}]_i$ in RBL-2H3 cells. Cytosolic free-calcium concentration ($[Ca^{2+}]_i$) in RBL-2H3 cells after 30 s of stimulation with 10–40 μ M of AA in the presence (◆) or absence (□) of external calcium was determined. The values are the mean $\pm$ S.D. for three different experiments. The $[Ca^{2+}]_i$ before stimulation was 60 nM.

Table 2

Effect of PUFAs on IP_3 formation in RBL-2H3 cells

Drug	IP_3 formation (pmol/ 4×10^5 cells) ^a
40 μ M AA	1.8 $\pm$ 0.5
40 μ M γ -LN	2.4 $\pm$ 0.7
40 μ M α -LN	1.8 $\pm$ 0.7
40 μ M EPA	1.9 $\pm$ 0.6
0.4% EtOH	1.7 $\pm$ 0.5

Each value represents the mean $\pm$ S.D. of triplicate determinations. Similar results were obtained in two other experiments.

^a Reaction was stopped at 20 min and IP_3 was extracted, and IP_3 concentration was measured by an IP_3 assay kit.

3.3. Effects of PUFAs on IP_3 formation in RBL-2H3 cells

Finally, we examined the effects of PUFAs on IP_3 formation in RBL-2H3 cells. As shown in Table 2, no changes in the IP_3 levels were observed after 20 min of incubation with 40 μ M AA. Furthermore, no changes in the IP_3 levels were observed when the RBL-2H3 cells were treated with 40 μ M γ -LN, 40 μ M α -LN or 40 μ M EPA. In addition, no significant changes were observed when the samples were treated with the ω -6 or ω -3 PUFAs. Therefore, the IP_3 level, which is probably determined by phosphatidylinositol turnover, and IP_3 formation are not involved in the ω -6 PUFAs-induced increase in $[Ca^{2+}]_i$.

4. Discussion

In the present study, we showed that ω -6 PUFAs specifically induced histamine release and a rapid increase in $[Ca^{2+}]_i$ in a cultured mast cell-like cell line, RBL-2H3. The specificity of the ω -6 PUFAs was confirmed, because neither the ω -3 PUFAs nor a ω -9 PUFA (OLE) were effective; two saturated fatty acids also did not have an effect.

Although some of the biological activities of PUFAs in the immune system are not distinguished among these groups of different ω -numbers [8], such as the triggering of cytosolic calcium mobilization in JURKAT T cells, some differences in their effects on the regulation of activated macrophage functions, like the production of interleukin 1 [9] or lymphocyte mitogenesis [10], have been observed. Furthermore, some differences in ω -6 and ω -3 fatty acids on the mastoparan-stimulated or IgE-mediated histamine release from a canine mastocytoma cell line (C2) or RBL-2H3 cell were reported [11–13]. In both cells, increase of degranulation by ω -6 fatty acid (AA) was higher than that by ω -3 fatty acid (EPA). The molecular mechanisms responsible for the regulation of mast cell functions by PUFAs are largely unknown. The greatest difference among PUFAs is their role as substrates for different series of prostaglandins (PGx); for example, ω -3 PUFAs are the sources of 1-series of PGx, like PGE₁ or PGA₁, whereas ω -6 PUFAs

are the sources of 2-series of PGx, like PGE₂ or PGA₂. In addition, before converting the AA to PGx, AA itself is released from RBL-2H3 cells in response to IgE or calcium ionophore stimulation [14]. Furthermore, in a previous paper, we showed that three days of pre-incubation with α -linolenic acid caused a reduction in AA levels and histamine release in RBL-2H3 cells [15]. We hypothesized that AA release itself, and not PGx, might have a biological effect on RBL-2H3 cells, leading to the regulation or even the initiation of subsequent reactions in the processes responsible for RBL-2H3 activation.

There was also an argument that fatty acids, especially PUFAs on both ω -3 and ω -6 series, regulate ion channels. In that report, AA was also suggested to act in an indirect way which involves cyclooxygenase and/or lipoxygenase products derived from AA [16–18]. In other cases, the arachidonic acid-induced Ca²⁺ release or Ca²⁺ influx, which were not inhibited by either cyclooxygenase or lipoxygenase inhibitors, have been reported [19,20]. The results of our study also showed that cyclooxygenase or lipoxygenase inhibitors did not inhibit the increase in [Ca²⁺]_i induced by AA. Thus, in RBL-2H3 cells, ω -6 PUFAs may regulate ion channels. So far as we examined, IP₃, a known mediator of [Ca²⁺]_i mobilization, was not produced in response to the stimulation with ω -6 PUFAs. Therefore, AA seems to mobilize cytosolic calcium via the same IP₃-independent mechanisms previously described in JURKAT T cells [8] or in stimulus-secretion coupling in pancreatic cells [21].

The activation of protein kinase C (PKC) by fatty acid, especially AA, has been reported in several cell lines [22]. In this paper, we showed that ω -6 PUFAs induced histamine release from RBL-2H3 cells. We previously reported that increases in [Ca²⁺]_i induced by a Ca²⁺-ATPase inhibitor and PKC activation caused synergistic histamine release from RBL-2H3 cells [23]. Therefore, it is plausible that PKC activation by AA or other ω -6 PUFAs may be induced in RBL-2H3 cells.

In conclusion, we showed that ω -6 PUFAs increased [Ca²⁺]_i and histamine release from RBL-2H3 cells, however, ω -3 PUFAs did not activate RBL-2H3 cells. These new findings are important for determining the mechanisms of the anti-allergic effects of ω -3 PUFAs.

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